Exhibit A

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Research Paper

Identification of Overexpression of Orphan G Protein-Coupled Receptor GPR49 in Human Colon and Ovarian Primary Tumors

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KEY WORDS

GPR49, G-protein-coupled receptor, colon tumors, ovarian tumors, overexpression

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ABSTRACT

We used gene expression profiling to probe differences in transcriptional output between 15 panels of colon tumor and matched normal colon tissues. This analysis revealed that GPR49, an orphan G Protein-Coupled Receptor (GPCR) is overexpressed in 66% (10/15) colon tumors compared with normal colon tissues. Subsequent analysis of an additional 39 sets of matched normal and tumor colon tissues by real-time quantitative reverse transcriptase confirmed the upregulation of this receptor. The differential expression of GPR49 between normal and tumor tissue was significant ($p > 0.001$). GPR49 was upregulated in 25 of 39 (64%) colon primary tumor tissues. In addition to colon tumors, GPR49 was also found to be upregulated in 18 of 33 (53%) ovarian primary tumor tissues analyzed by RT-PCR. Moreover, the expression level of GPR49 in colon and ovarian tumors increased in more advanced tumors suggesting a role for the receptor in tumor progression. The selective overexpression of GPR49 in tumor tissues was further illustrated by specific immunohistochemical staining of colon and ovarian tumor tissues, a finding that correlates with the mRNA expression of the receptor. In addition, expression of GPR49 induced transformation in a ligand-dependent manner and Knockdown of GPR49 mRNA level induced apoptosis in colon tumor cells. These novel findings provide a foundation for further studies and suggest a potential role for GPR49 in tumorigenesis.

INTRODUCTION

Colorectal cancer is a leading cause of cancer death in the United States.[1] While there have been improvements in the outcome of patients with colorectal cancer through various therapeutic approaches such as the use of cytotoxic chemotherapy, surgical resection and monoclonal antibody therapy; patients with advanced colorectal cancer await more effective therapeutic drugs.

Models explaining the development of colorectal cancer suggest that tumorigenesis is multi-step process driven by genetic alterations in a variety of genes that regulate cell growth, proliferation and DNA repair. These models suggest that colorectal cancer develops as a result of progressive accumulation of genetic changes, eventually leading to the transformation of normal colon tissue to colon adenocarcinoma. Analysis of molecular genetics of colorectal cancers revealed that colon cancer is commonly initiated by alterations of signal transduction pathways that regulate cell growth followed by genetic changes that activate oncogenes or inactivate tumor suppressor genes.[2] These genetic alterations likely represent necessary steps in the development of colon cancer. The genes implicated in colorectal tumorigenesis include KRAS, APC, and P53. Most recently, overexpression of PRL-3, a tyrosine phosphatase and mutations in B-RAF were observed in some colon cancer tissues.[3,4] Since K-Ras is frequently mutated in colon tumors, the identification of B-RAF in these tumors underscores the critical role of activation of the RAS-RAF-MEK pathway in this disease. Despite the progress made in understanding the molecular basis of colon tumor formation, identification of novel genes that contribute to colon cancer development is important for complete characterization and ultimately treatment of colorectal cancers.

Gene expression profiling is a powerful technique that has been used to monitor global patterns of gene expression in tumor versus normal tissue.[5-8] In this study, we used microarray profiling to examine gene expression differences between normal and malignant colon tissues. We report the novel finding of upregulation of the orphan G protein-coupled receptor 49 (GPR 49) in colon tumors. The gene was upregulated (>4-fold) in 10 out of 15 colon tumor samples compared to the matched normal controls. The elevated level of expression of this gene was confirmed by real-time quantitative reverse transcriptase analysis

of tumor and matched normal adjacent tissues from patients with colorectal cancer. In addition to colorectal tumors, overexpression of GPR49 was observed in primary ovarian tumors. Furthermore, we confirmed overexpression of GPR49 by immunohistochemical analysis of colon and ovarian tumor tissues compared to normal controls. Interestingly, the expression level of GPR49 increases with disease progression. This suggests a role for the receptor in latter-stage colorectal tumors. Ectopic expression of GPR49 in fibroblast results in focus formation. In addition, small interfering RNAs that specifically knock down expression of GPR49 in colon tumor cell line induced apoptosis. Taken together, these novel findings suggest a role for the receptor in tumorigenesis.

Table 1 **Transcripts more highly expressed in colorectal tumors than in normal tissues**

Description	Accession #	Fold upregulation	# of tumor in which gene is upregulated/total tumor
Human sarcosin mRNA	AF056929	9.6	(3/15)
Human mRNA for titin	X90568	9.4	(2/15)
Human desmin mRNA	HSU59167	9.3	(3/15)
Human carbonic anhydrase III	HUMCAIII7	9.2	(1/15)
Human beta-thromboglobulin-like protein	M17017	9	(2/15)
Human mRNA for calcium-binding protein S100	X65614	8.8	(4/15)
regenerating protein	BAA03111	8.6	(3/15)
Human XAGE-1 mRNA	AF251237	8.5	(1/15)
Human osteonectin gene	M25743	7.7	(2/15)
Human cDNA FLJ13153	AK023215	7.7	(3/15)
Human mRNA for prepro-alpha1(I) collagen	Z74615	7.5	(4/15)
Human cytokine (GRO-beta)	M36820	6.4	(4/15)
Human mRNA for matrix Gla protein.	X53331	6.2	(3/15)
Human cDNA: FLJ22774	AK026427	6.2	(2/15)
Human E2IG1 (E2IG1) mRNA	AF191017	6	(3/15)
Human insulin-like growth factor I (IGF-1)	M37483	5.5	(4/15)
Human gene for heat shock protein 40	D85429	5	(3/15)
Human mRNA for KIAA0332 gene	AB002330	5	(2/15)
Human mRNA for Rap1B	X08004	5	(4/15)
Human vascular endothelial cell growth factor	AF016098	4.7	(3/15)
Human Rad mRNA	L24564	4.7	(2/15)
Human metalloproteinase (HME) mRNA	L23808	4.5	(1/15)
Human orphan G-protein coupled receptor	AF062006	4.4	(10/15)
Human A-kinase anchoring protein	AF176555	4.2	(3/15)
Human melanoma growth stimulatory protein	U03019	4	(2/15)
Human claudin-1 (CLDN1) mRNA	AF134160	4	(3/15)
Human desmin gene	M63391	2.4	(3/15)
Human mRNA for KIAA0511 protein	AB011083	2.4	(2/15)
Human secreted frizzle protein	AF056087	2.2	(3/15)
Human mRNA for KIAA1489 protein	AB040922	2.2	(4/15)
Human NF-kB-activating kinase NAK	AF174536	2	(3/15)
Human mRNA for elongation factor	X60489	2	(2/15)
Human Wee1 gene	X62048	2	(2/15)

RESULTS

Overexpression of G protein-coupled receptor 49 mRNA in primary colorectal tumor tissues by cDNA microarray. Differences in gene expression between normal colon tissue and colon adenocarcinoma were detected using microarrays consisting of 68,873 cDNA clones. The relative expression of each gene was compared between fifteen colon tumor tissues and their corresponding matching normal tissues. We classified a gene as overexpressed only when it showed greater than three fold differences in expression in at least 5 tumor samples. One gene that was highly upregulated in the majority of primary colorectal tumor tissues by this analysis was an orphan G-Protein coupled receptor 49 (GPR49). This gene was upregulated (3.5–4.4-fold) in 11 out of the 15 primary colorectal tumor tissues compared to their normal matched control samples (see Table 1). Most upregulated genes displayed less than two fold upregulation or the upregulation was not considered significant because it was seen in less than four tumor samples.

Overexpression of GPR49 in colon tumor cell lines. To confirm the differential upregulation of detected by microarray, we performed real-time quantitative PCR analysis of GPR49 expression in colon tumor cell lines and nontumor cell lines. cDNAs were generated from each sample and used as templates for quantitative PCR using GPR49 specific primers. Parallel PCR reactions that detected a housekeeping gene were used as control. Minimal differences in the control gene were observed between normal and tumor samples. The mRNA expression level of GPR49 is highly upregulated in colon tumor cell lines compared to CRL-1459, a human colon endothelial cell line that is nonmalignant (Fig. 1A). Seven out the ten colon tumor cell lines displayed elevated levels of GPR49 expression. The average cycle threshold (Ct) values for normal colon cells and tumor cells were 40 and 26 respectively. As shown in (Fig. 1A), in addition to the colon tumor cell lines, the fetal normal colon cell line CRL-1831 also exhibited high expression of GPR49 although to a lesser extent. The fold upregulation of GPR49 observed in the seven colon tumor cell lines ranged from 150 to 9000 fold. The expression pattern of GPR49 mRNA in several normal tissues was also analyzed and was found to be expressed in placenta and skeletal muscle but was low or undetectable in other tissues (Fig 1B). This observation is in agreement with published data showing the restricted expression of GPR49 mRNA in normal tissues.[9] The expression of GPR49 mRNA observed in placenta and skeletal muscle is much lower than that in the colon tumor cells (Fig 1A).

We expanded the analysis of the expression profile of GPR49 to include the NCI60 panel which contains cell lines derived from cancers of several tissue types. Figure 1C shows the expression profile of GPR49 in tumor and normal cell lines. In addition to colon tumor cell lines the elevated expression

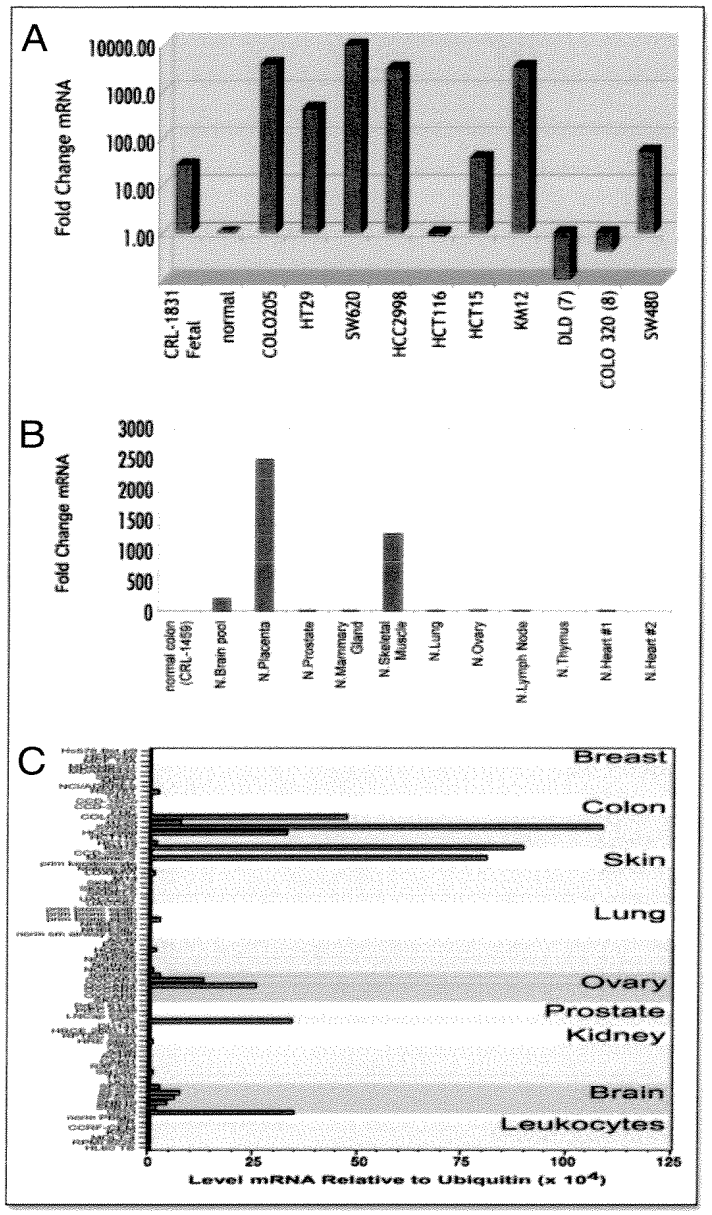

Figure 1. Expression profile of GPR49 in colon tumor cell lines and normal tissues. (A) Colon tumor cell lines, non-tumor colon cell lines CRL-1459 and CTRL-1831 (colon fetal cell line) were grown to confluence and total RNA was isolated. The mRNA level of GPR49 was measured by real-time quantitative reverse transcriptase-PCR (Taqman) using a PE Applied Biosystems Prism model 7700. The Expression of GPR49 was normalized to 23 kD highly basic protein. Expression of GPR49 in tumor cells is shown as fold elevation over that of nontumor colon cell line CRL-1459. (B) Expression of GPR49 in various normal tissues was analyzed as above. Expression of GPR49 is shown relative to nontumor colon cell line CRL-1459. GPR49 mRNA was found be expressed mainly in placenta and skeletal muscle tissues. (C) A panel of tumor and normal cell lines were analyzed for GPR49 expression by quantitative RT-PCR analysis. The expression of GPR49 was normalized to ubiquitin. The fold upregulation of GPR49 mRNA relative to ubiquitin is shown. GPR49 mRNA is upregulated in colon, ovary, glioblastoma and prostate tumor cell lines. The yellow shaded columns represent the normal cell lines and the gray shaded columns represent the tumor cell lines. There are no normal samples for the ovary and brain cell lines. The normal cell lines that were used include primary Keratinocytes, primary bronchial epithelial and normal cell small airway epithelial.

level of GPR49 was detected in ovarian tumor cells, a prostate cell line (PC3), a melanoma cell line (Malme-3) and some glioblastoma cell lines. Upregulation of GPR49 was not observed in breast and lung tumor cell lines, when compared to their respective nontumor cell lines. The expression data from these tumor cell lines suggest that GPR49 is selectively upregulated in colon, ovarian and glioblastoma tumors.

Overexpression of GPR49 in primary tumors. Having confirmed the upregulation of GPR49 mRNA in colon tumor cell lines, we investigated the overexpression of GPR49 in primary colon tumor tissues. Quantitative RT-PCR analysis was performed on 39 sets of matched normal tissue and colon tumor (pathologically staged I–IV) tissues obtained from clinical sources and 11 samples of colon tissues obtained from donors who did not have cancer. These 11 samples together with the matched normal adjacent colon tissues from the cancer patients were used as control groups to monitor the expression level of GPR49 in colon tumors. The results of this analysis Figure 2A showed that normal colon tissue samples had low or undetectable (Ct = 40) level of GPR49 mRNA expression. In contrast, overexpression of GPR49 mRNA was observed in 25 of the 39 (64%) colon tumor tissues (mean Ct = 25). These observations correlate with the overexpression of GPR49 seen in the microarray and colon tumor cell lines analysis.

We next investigated whether the expression level of GPR49 changed with the histological grade of the tumors. The colon tumor samples were grouped by stage, (based on tumor size, invasiveness and metastasis) and the expression level of GPR49 was monitored. The data in Figure 2B show that the expression of GPR49 was found to be highest in tumor stages I and II using Kruskal-Wallis and Dunn's multiple comparison test. The fold difference in GPR49 expression between the control group and stages I and II is 4 and 13 fold respectively.

We also examined the expression levels of GPR49 in ovarian tumors. RNA was extracted from ovarian cancer tissues and primary tissues and subjected to quantitative RT-PCR to determine the expression profile of GPR49. Elevated expression of GPR49 was observed in 18 of the 33 (53%) ovarian tumor samples compared to normal ovarian tissues (Fig. 2C). We examined the correlation between tumor stage and the expression level to determine whether the expression of GPR49 increases in later stages of the tumors. GPR49 expression was found to be statistically different from control in stage I and II ovarian cancer, but not in stage III and IV ($p < 0.001$, Fig. 2D).

Overexpression of GPR49 protein in colorectal and ovarian tumor tissues. The upregulation of GPR49 in colon and ovarian tumor tissues was further evaluated using by immunohistochemical analysis. Immunohistochemical staining was performed on primary colon and ovarian tumors and normal tissues using two antibodies raised against different regions of the GPR49 protein sequence (Fig. 3A and B). Immunostaining with the anti-GPR49 antibodies showed an intense and specific staining in all 14 of the colon tumor samples and 8 of the 9 ovarian tumor samples that were analyzed. The staining was absent or very weak in the noncancerous colon and ovarian tissues. In the colon tumor tissues the epithelial cancer cells were strongly positive. Adjacent normal colonic mucosa was negative. Strong staining of the tumor cells in the ovary tumor was also detected. In the ovary tumor samples, the capillary endothelial cells were also very strongly stained. The specific staining was detected with both antibodies in the colon and ovarian tumors tissues providing collaborative data. The staining results of representative samples of each tumor type, together with adjacent normal tissues are shown in Figure 3. These observations demonstrate that GPR49 protein is highly expressed in colon and ovarian tumors.

Expression of GPR49 in fibroblasts results in focus formation in the presence of conditioned medium. We wanted to assess the consequences of expression of GPR49 on cell growth. Expression plasmids containing full-length GPR49 gene was expressed in NIH3T3 cells and focus formation was monitored. Expression of GPR49 failed to induce focus formation after four weeks in culture under standard conditions-5% fetal calf serum (data not shown). Because GPR49 is an orphan G protein-coupled receptor whose ligand is unknown, we reasoned that GPR49 might transform in a ligand dependent manner. Previous studies have shown that some G protein coupled

receptors transform NIH3T3 cells in a ligand dependent manner.[10] The observations that GPR49 is overexpressed in colon tumors, led us to hypothesize that its ligand could also be expressed in colon tumors. Therefore, plasmids expressing GPR49 wild type, or empty vector were transfected in NIH3T3 cells and the transfectant cells were supplemented with conditioned media from colon tumor cell lines. Within three weeks, cells transfected with GPR49 formed foci in the presence of conditioned medium (Fig. 5A). Focus formation was not observed in the untransfected NIH3T3 cells or in cells transfected with expression vector alone when grown in the presence of conditioned medium. Focus formation was observed in cells transfected with activated H-ras in the absence of conditioned medium (used as a positive control). These observations suggest that GPR49 can transform NIH3T3 cells in a ligand dependent manner.

Knockdown of GPR49 induces apoptosis. Next, we evaluated the effect of GPR49 downregulation in SW620 cancer cells using siRNA knockdown. We designed specific siRNA for GPR49 and evaluated their ability to knockdown GPR49 mRNA (we were unsucessful in generating antibodies against GPR49 that worked in western blots hence we could not evaluate the knockdown at the protein level). siRNA oligo GPR49-2 which showed the highest knockdown of GPR49 mRNA was selected to investigate cell cycle distribution in human colon tumor cells after knockdown of GPR49. As shown in Figure 5B, 16% of the cells transfected with GPR49 siRNA were in Sub G_1 phase (an indicator of cells undergoing apoptosis) compared to only 3% of cells transfected with scrambled control siRNA. Similar results were observed in Colo-205 and HCC298 cell lines (data not shown). Induction of apoptosis was further assessed by immunoblot analysis of PARP cleavage (an apoptotic marker) in cells transfected with GPR49 siRNA or control siRNA. As illustrated in Figure 5C, knockdown of GPR49 induced significant increase in the level of cleaved PARP compared to cells transfected with control siRNA. This result indicates that inhibition of GPR49 expression can induce apoptosis in human colon cancer cells.

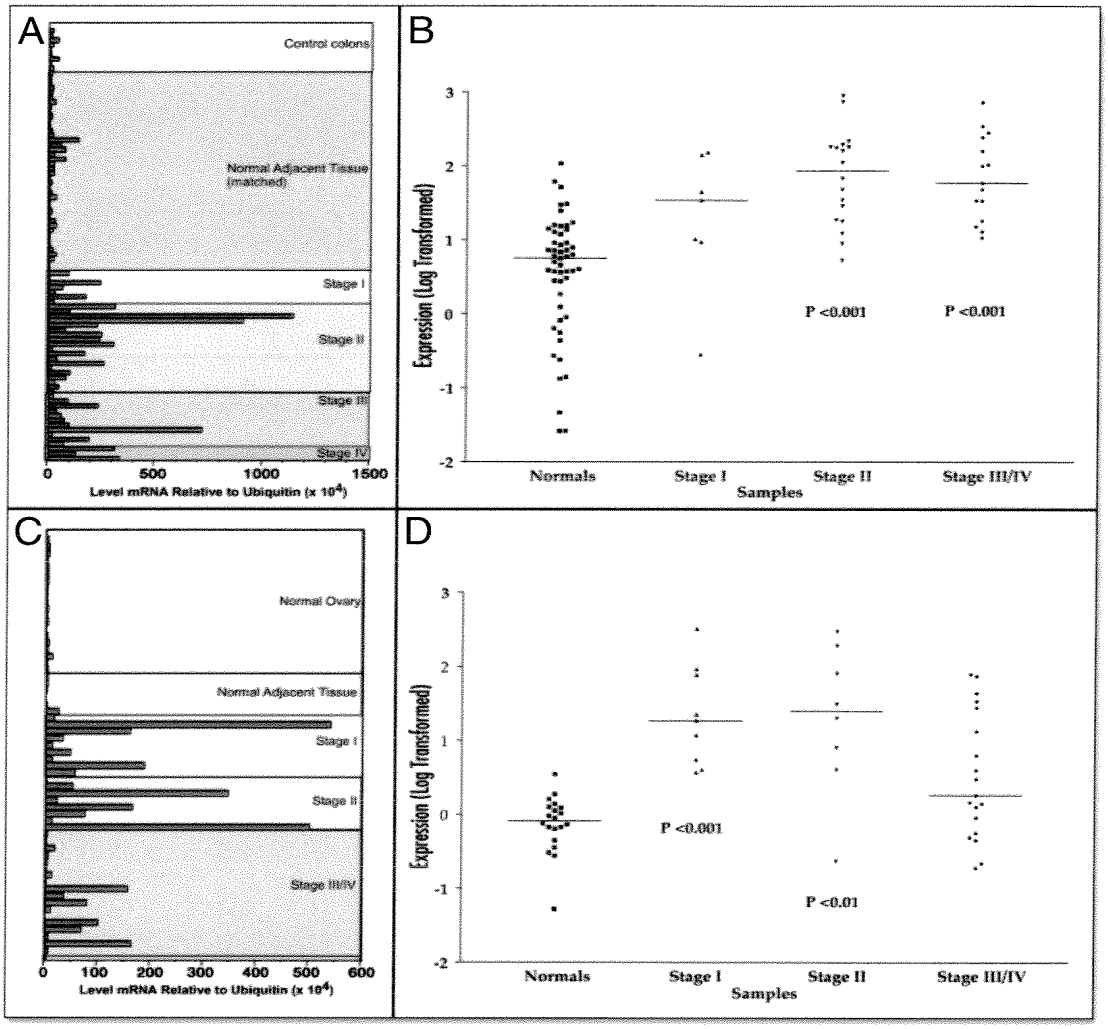

Figure 2. Expression analyses of GPR49 in primary colorectal tumors from patients. (A) Quantitative real-time RT-PCR mRNA expression of GPR49 in colon tumor and matched normal adjacent tissues from 39 patients and colon tissue samples from 11 donors who did not have cancer is shown as fold increase. The fold upregulation of GPR49 mRNA relative to ubiquitin is shown for each tumor sample tested. The samples are grouped into colon controls (colon tissues samples from patients with no cancer), normal matched adjacent tissue samples, and the colon tumor samples grouped into pathological stages. Expression of GPR49 is highly upregulated in the tumor tissues compared to the normal tissues. (B) Expression level of GPR49 increases in primary colorectal tumors. The tumor samples are grouped by stage, which depends on tumor size, invasiveness, and metastasis. Statistical analysis revealed a significant difference in GPR49 expression between the controls and the colorectal adenocarcinomas ($p < 0.001$ using the Kruskal-Wallis non-parametric test). The fold differences of the means relative to the control group are: stage I, 4 fold, stage II, 13 fold, stage III, 10 fold and stage IV: 30 fold. (C) Expression of GPR49 in ovarian tumors. Ovarian tissue samples from 26 patients and samples from normal ovary tissues were probed for GPR49 expression by quantitative RT-PCR analysis. The expression of GPR49 in ovarian tumor and normal tissues relative to ubiquitin are shown. The tumor samples show an elevated expression of GPR49. (D) Expression level of GPR49 increases in primary ovarian tumors. The tumor samples are grouped by stage, which depends on tumor size, invasiveness, and metastasis. Statistical analysis revealed a significant difference in GPR49 expression between the normal and the tumor ovarian tissues ($p < 0.001$ using the Kruskal-Wallis nonparametric test).

DISCUSSION

We initially identified the upregulation of GPR49 mRNA using expression profiling of ten colon tumors compared with matched normal tissues. Based on the microarray data, GPR49 mRNA was upregulated in 70% of colon tumors. We further tested the overexpression of GPR49 by real-time quantitative RT-PCR analysis of a

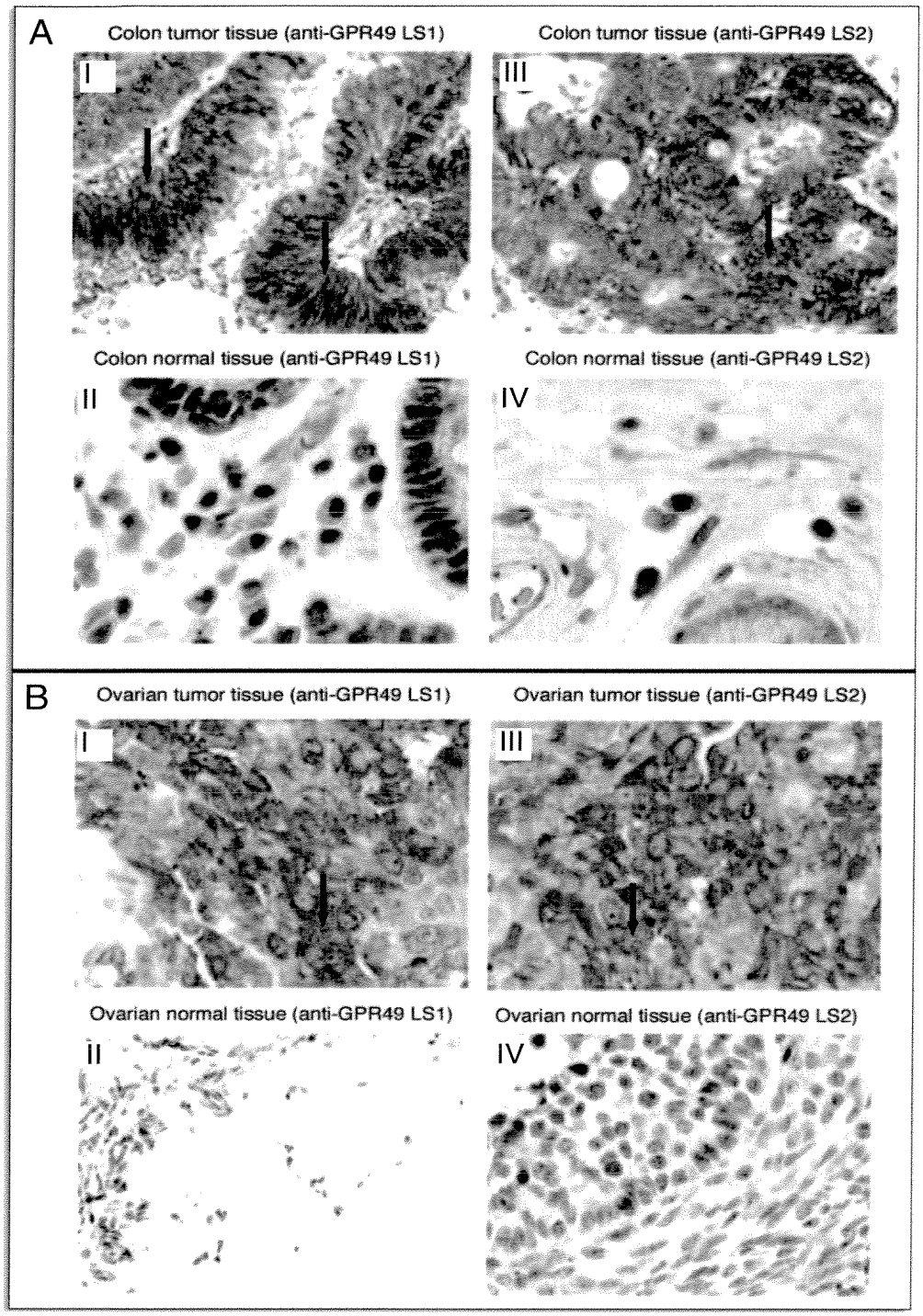

Figure 3. Immunohistochemical analyses of GPR49 expression in human colon and ovarian tumors. (A) Low-power image (40X) of anti-GPR49 staining of four representatives paraffin-embedded sections of tumor (I and III), and normal (II and IV) colon epithelial tissues and tumor ovarian epithelial tissues stained with affinity purified anti-GPR49 antibodies are shown (positive cells are stained red, purple/blue staining is hematoxylin counterstain to visualize tissue architecture). Two different antibodies (antibody LS1 and LS2) generated against different regions of GPR49 were used in the immunohistochemical analyses. The colon tumor tissues showed a high intensity staining (red stain in I and III) whereas staining was absent or weak in the normal colon tissue (II and IV). (B) staining of tumor (I and III) and normal (II and IV) of ovarian tissues using anti-GPR49 antibodies as described above. Strong staining (red) was detected in the tumor samples (I and III) as compared to the normal tissues (II and IV). A total of 14 colon tumor and normal tissues and eight ovarian tumor and normal tissues were examined and the results are similar to what is shown.

broad panel of colon and ovarian tumor tissues from patients as well as in tumor cell lines. The results obtained from these analyses demonstrate that GPR49 transcript level is upregulated in colon and ovarian tumors. Interestingly, analysis of the Cancer Gene Anatomy Project database (CGAP) which contains expressed sequences from normal and tumor cDNA libraries[11] also demonstrate the overexpression of GPR49 in colon and ovarian tumors, (data not shown). This observation is in agreement with our data. When we grouped the colon and ovarian tumors by stages, we observed that the expression level of GPR49 was high in advanced tumors compared to the control groups. For example, the fold overexpression of GPR49 mRNA level between the control groups (normal adjacent tissues) and Stage I and Stage II of primary colorectal tumors was 4-fold and 13-fold, respectively. However, at stages III/IV the expression of GPR49 seems to decrease (in both colorectal and ovarian tumors) suggesting that the overexpression of GPR49 may be an early event in tumorigenesis. In addition to mRNA upregulation, GPR49 protein was immunohistochemically detected in primary colon and ovarian tumor tissues and was absent or very weakly detected in the corresponding non-cancerous tissues. Moreover, both antibodies which were generated from different regions of GPR49 protein stained strongly the colon and ovarian tumors, while the corresponding non-tumor specimens showed weak staining. These results further demonstrate the overexpression of GPR49 protein in primary colon and ovarian tumors.

The ability of GPR49 to induce transformation of NIH3T3 in the presence of conditioned media from colorectal tumor cells suggests a role for the receptor in growth regulation. The requirement for conditioned media suggests that transformation occurs in a ligand-dependent manner. These findings indicate the possibility that the ligand for GPR49 could be secreted by the tumor cells, suggesting a paracrine/ autocrine mechanism of action. Indeed, such a

mechanism of action has been previously reported for the neuropeptide and prostaglandin receptors.[12] The observation that siRNA knockdown of GPR49 in colon cancer cells results in apoptosis suggests that GPR49 may inhibit induction of apoptosis in colon tumor cells.

G protein coupled receptors represent the largest family of transmembrane receptors responsible for signal transduction from a variety of extracellular factors.[13] Ligand binding to the G protein coupled receptor.

results in activation of intracellular heteromeric G proteins to induce downstream signaling. The characteristic motif of this superfamily is the presence of seven membrane-spanning domains and an extracellular N-terminal portion of variable length and intracellular C-terminus.[14] GPR49 is an orphan receptor that belongs to the family of glycoprotein hormone receptors that include gonadtropin and thyrotropin receptors.[9,15] These glycoprotein hormone receptors have a large extracellular domain with leucine-rich repeats to which the glycoprotein ligand binds. The ligand and function of GPR49 are unknown, however, the presence of a large extracellular domain with leucine-rich repeat motifs suggest that its ligand is probably a glycoprotein. The functional role of GPR49 is not well understood. However, a recent study shows that targeted deletion of GPR49 gene in mice results in neonatal lethality which appears to be due to gastric distension and ankyloglossia.[16]

G protein coupled receptors are increasingly implicated in neoplastic transformation and have been found in a number of tumors. In some cases these receptors have been shown be amplified, overexpressed or acquired a gain-of-function mutation in tumors. These findings suggest a link between these receptors and tumor cell proliferation. Examples of growth stimulatory role of the GPCRs in tumor cells are demonstrated by the growth regulation of small cell lung carcinoma tumor cells by neuropeptides acting on cognate GPCRs.[17] Intriguingly, GPCRs have been shown to be oncogenic and to induce transformation, making these receptors a novel class of transforming oncogenes. Such examples are the *mas* GPCR which can induce formation of tumors in mice, and the muscarinic receptors shown to transform NIH3T3 cells in a ligand-dependent manner.[18] In addition, constitutive active mutant GPCRs have been isolated from various human tumors. Thyroid stimulating hormone receptor (TSH) and leutinizing hormone receptor (LH) which belong to the GPR49 subfamily, have been identified in thyroid adenomas and hyperplastic growth of Leydig cells respectively.[19] These findings strongly implicate G protein coupled receptors in tumorigenesis.

A major question that arises from this study is what is the role GPR49 in tumorigenesis? Our data strongly demonstrate the selective upregulation of GPR49 in colon and ovarian tumors, suggesting a correlation with the disease. The role of GPR49 in tumorigenesis is supported by its induction of transformation when it is expressed in NIH3T3 cells. The ability of GPR49 to transform and its selective elevated expression in colon and ovarian tumors suggest relevance of the receptor to mechanisms that operate and drive cancer pathogenesis.

The upregulation of GPR49 in tumors makes it an attractive target for the treatment of colon and ovarian tumors. A recent study reported the specific overexpression of GPR49 in human hepatocellular carcinomas where GPR49 overexpression correlated with activating mutations in the wnt/beta catenin pathway suggesting that GPR49 may play a role in colon cancer, where activating mutations of the wnt/beta-catenin pathway are frequently observed.[20] However, some of the cell lines used in our study overexpress GPR49 but do not have activating beta catenin mutations, suggesting that beta catenin mutation does not correlate with GPR49 upregulation.

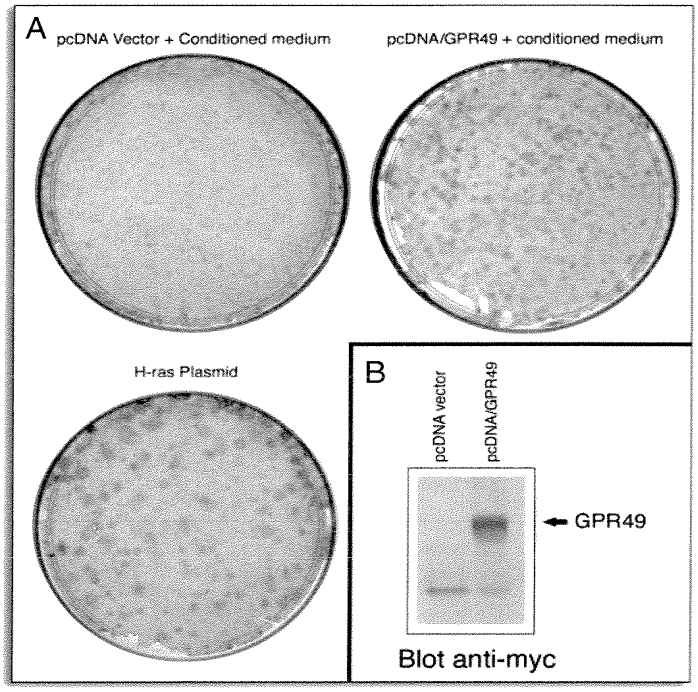

Figure 4. GPR49 transforms NIH3T3 cells in a ligand dependent manner. (A) analysis of transformating potentials. pcDNA3 empty vector (mock), pcDNA3-GPR49 and pSport-H-ras activating mutant (positive control) were separately transfected into NIH3T3 cell lines. The transfectants (except for the ras transfections) were supplemented with conditioned medium (75% medium from SW620 cell lines and 25% DMEM containing 5% calf-serum). To examine foci formation, the cells were stained with xylene staining. The dark spots are xycline-stained foci indicating transformation. (B) total cell lysate (40 µg) from pcDNA vector and pcDNA/GPR49 plasmid (myc tagged) transfections were analyzed by an immunoblot using antibodies directed against myc epitope to confirm the expression of GPR49.

In conclusion, we demonstrate the significant upregulation of G protein coupled receptor 49 in colon and ovarian tumors. Although the function of GPR49 is not well characterized, the novel findings outlined in this study implicate it in tumorigenesis. These findings suggest that the receptor is a potential new molecular target and a biomarker in colon and ovarian tumors. Experiments to identify the ligand for GPR49 and the elucidation of its function are currently underway.

MATERIALS AND METHODS

Differential expression analysis. Differential expression was generated using Incyte's Human GeneAlbum microarrays consisting of 65,873 cDNA clones representing 33,515 individual genes. RNA was isolated from 15 samples of primary colon cancers and matched normal tissue and fluorescent labeled with Cy3 and Cy5. The labeled samples were then run in hybridizations across cDNA microarrays. The data from a minimum of four donors was averaged together to generate the differential expression ratios. Significant differential expression was defined as greater than a three-fold change.

Human tissue specimens and cell lines. Human colorectal cancer tissues were obtained from Princess Alexandra Hospital Tumor Tissue Bank (26 cases) (Woolloongabba, Queensland, Australia) and Zoion Diagnostics (26 cases) (Shrewsbury, MA). Human ovarian cancer tissues were obtained from Zoion Diagnostics (63 cases). Tumor tissue was collected for each case, and when possible matching normal adjacent tissue was also collected. Each case was

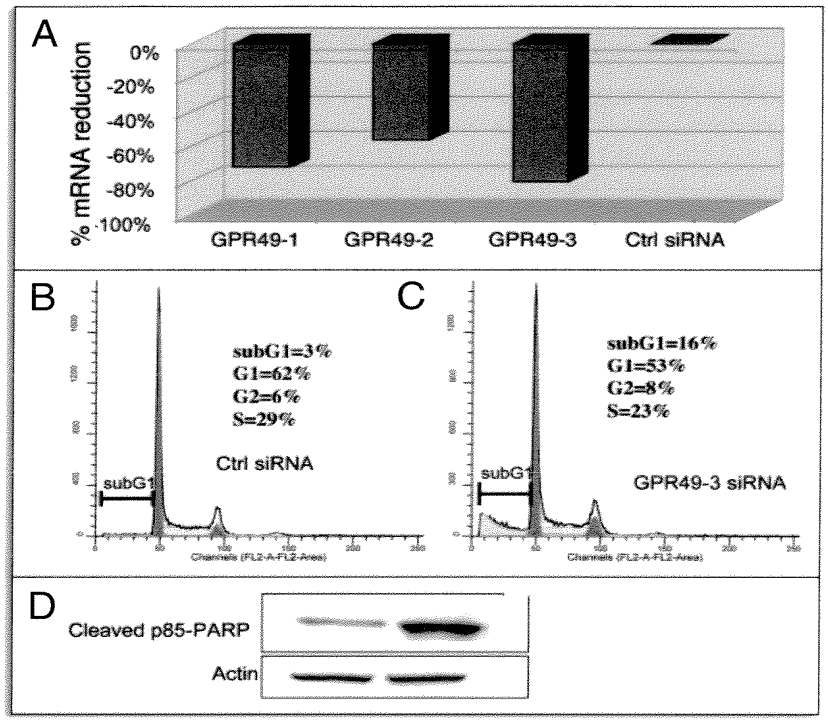

Figure 5. Knockdown of GPR49 induces apoptosis. (A) 50 nm of GPR49 specific siRNA oliogos and a control siRNA oligo were transfected in SW620 colon cell lines, cells were collected 72 hours after siRNA transfection and subsequently assayed for GPR49 mRNA level using real-time PCR. (B) Non-targeting siRNA control or GPR49 specific siRNA were transfected into SW620, cells were collected 72 hours after siRNA transfection and subsequently assayed for their DNA content by flow cytometry. Percentage of cells in sub G_1, G_1, G_2/M and S phase is depicted. These experiments were performed three independent times and representative results are shown. (C) SW620 tumor cells were transfected with control siRNA or GPR49 specific siRNA and after 72 hours, cells were harvested and probed for cleaved PARP. (D) The same western blot was reblotted with anti-actin antibody as a control for protein loading.

accompanied with a brief clinical history, pathology report, and pathological diagnosis. This information was used to select cases, 39 colorectal and 34 ovarian cases, to be studied for expression analysis. All colorectal cancers included in this study were confirmed to be adenocarcinomas and all ovarian cancer cases were confirmed to be papillary serous cystadenocarcinoma by an in-house pathologist. Cases excluded from the study had either other diagnoses or large areas of necrotic tissue. Two types of control tissues were used in this study, normal adjacent tissues collected from the above described cases as well as additional control tissues obtained from the NDRI (Philadelphia, PA). NDRI provided normal adjacent tissues from two additional colorectal cancer cases, three normal colon samples, five colon samples from patients with diverticulosis, and 20 normal ovary samples from patients undergoing hysterectomy. All tissues included in this study were obtained from surgical procedures that patients were undergoing as treatment for medical conditions and were frozen in liquid nitrogen within approximately one hour of excision. TNM staging information provided in the pathology reports was used to group the samples for analysis of the data.

The cell lines used in the study were obtained grown in RPMI 1640 medium containing 5% fetal bovine serum (Life Technologies, Inc.). Cells (~5 x 10^7) were harvested during exponential growth phase in 15 mls of guanidine isothiocyanate solution containing sarkosyl, β-mercaptoethanol, and antifoam A. Total RNA was purified from the GITC lysate using a cesium chloride gradient centrifugation technique followed by phenol/chloroform extraction and ethanol precipitation. Total RNA was treated with DNase I (Boehringer-Mannheim) in the presence of RNAsin (Promega) to remove any traces of genomic DNA contamination prior to cDNA synthesis. cDNA was synthesized with a mixture of random hexamer and oligodeoxythymidylic acid primers using Superscript II reverse transcriptase (Life Technologies, Inc.) in the presence of RNasin.

Statistical Analyses. Thresh hold values (Ct) were generated for GPR49 and a housekeeping gene (Ubiquitin) on the ABI 7900HT sequence detector. These values were inserted into the equation $(1.8^{(Ct\ housekeeping\ gene\ -\ Ct\ gene\ of\ interest)})$ to generate a delta Ct value that is normalized to the housekeeping gene. The values generated were multiplied by 10,000 to get a nondecimal number. The Log_{10} of these values were used in the statistical analysis.

The human tissues samples were divided into clinical groupings (controls, stage I tumors, stage II tumors, etc.), graphed in GraphPad Prism and analyzed by a one-way ANOVA (Kruskal-Wallis test and a Dunn's Multiple Comparison Test) to determine the significant differences between the means of the different groups.

Real-Time quantitative PCR for gene expression. DNase-treated total RNA was reverse-transcribed using Superscript II (Gibco/BRL) according to manufacturer's instructions. Primers were designed using Primer Express (PE Biosystems, Foster City, CA). Real-time quantitative PCR on 10 ng of cDNA from each sample was performed using either of two methods. In the first method, two GPR49-specific unlabelled primers were utilized at 400 nM in a Perkin Elmer SYBR green real-time quantitative PCR assay utilizing an ABI 5700 instrument. In the second method, two unlabelled primers at 900 nM each were used with 250 nM of FAM-labeled probe (Applied Biosystems, Foster City, CA) in a Taqman™ real-time quantitative PCR reaction on an ABI 7700 sequence detection system. The absence of genomic DNA contamination was confirmed by using primers that recognize genomic region of the CD4 promoter. Ubiquitin levels were measured in a separate reaction and used to normalize the data. (Using the mean cycle threshold value for ubiquitin and GPR49 for each sample, the equation 1.8 e (Ct ubiquitin minus Ct GPR49) x 10^4 was used to obtain the normalized values.) Statistical analysis of tumor expression was conducted using a Kruskal-Wallis nonparametric test.

Cloning of full-length human GPR49. Full-length human GPR49 was generated by polymerase chain reaction using CLONTECH's Advantage 2 Polymerase Mix. Template cDNA was prepared as explained above from SW620 colon cell lines which were shown by Taqman analysis to overexpress GPR49. Cloning primers were designed to introduce a Kpn1 restriction enzyme site on the 5' end while retaining the start codon. The 3' cloning primer introduces an Xba1 restriction enzyme site which eliminates the stop codon. Primer sequences are listed below; highlighted nucleotides are substitutions creating restriction sites. The resulting full-length GPR49 product was cloned into the Kpn1 and Xba1 sites of the pcDNA3.1/myc-His expression vector (Invitrogen). 5' primer sequence CCTACTTCGGGTACCATGGACACCTCCC 3' primer sequence TTAA-TTCTAGACATGGGACAAATGCCACAGAGG. A wild-type human GPR49 construct was generated from the variant obtained from the SW620 cell line by utilizing the QuickChange Site-Directed Mutagenesis method (Stratagene). A set of complimentary oligonucleotides was generated with the following sequence AGCGTGGGTTCTCTGTGAAATATTCTGCAAA. All sequences were confirmed using the Beckman Coulter CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit.

Immunohistochemistry. Immunohistochemical staining for GPR49 was performed by LifeSpan BioSciences (Seattle, WA). Briefly, paraffin-embedded tissues, which included colon and ovarian tumor and normal tissues, were incubated with primary antibodies against GPR49 (generated by LifeSpan BioSciences). Sections were counterstained with hematoxylin and mounted in DPX mountant (Sigma). Appropriate negative controls were performed by omitting the primary antibody and/or substituting it with an irrelevant antiserum.

Focus formation assays. Twenty-four hours before transfection, the NIH3T3 cells were plated at a density of 10^5 cells onto 60 mm plates and transfected with 1 μg plasmid DNA encoding each expression construct using a modified Fugen 6 method. Eighteen hours after transfection, the cells were refed with DMEM containing 10% calf serum. For the transformation assay the cells were refed 8 h later with DMEM containing 5% calf serum, or conditioned medium (75% medium from growing SW620 cells and 25% DMEM medium. The medium from SW620 cells was centrifuged and filtered. Foci formation was scored after 20 days. The focus formation assay was performed in three separate experiments.

Western blot analysis. Proteins were extracted with cell lysis buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM benzamidine, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μM okadaic acid, and 1% Nonidet P-40. Proteins were resolved on 10% SDS-polyacrylamide gels, transferred to nitrocellulose filters and blotted with anti-myc antibody (Bohringer). Western blots were developed using enhanced chemiluminescence (Amersham Biosciences). Anti-rabbit IgG peroxidase-conjugated antibodies were used as secondary antibodies.

Small interfering RNA. Small interfering RNAs (siRNA) were synthesized by Xeragon (Qiagen) and Sequiter (Invitrogen). The sequence of siRNA GPR49 targeting nucleotides were (GPR49-1) CTTGTTCAATCCTCACTTT, (GPR-2) GATCTGTCTTACAACCTAT, (GPR49-3) CTAGGATTTCATAGCAACA, siRNAs were dissolved in buffer (100 mM potassium acetate, 30 mM HEPES-potassium hydroxide, and 2 mM magnesium acetate, pH 7.4) to a final concentration of 20 μM. The siRNA solutions were heated to 90 °C for 60 s and incubated at 37°C for 60 min prior to use to disrupt any higher order aggregates formed during synthesis. 2 x 10^6 cells were plated into 100 mm plate and allowed to adhere for 24 h. Sixteen microliters of Lipofectamine transfection reagent (Invitrogen Inc.) and 50 nM of siRNA oligos were mixed in an eppendorf containing 500 microliters of serum-free DMEM media and incubated at room temperature for 30 min. The transfection agent/siRNA complex was added into the plates containing eight milliliters of DMEM with 10% FBS and incubated in normal cell culture conditions for 72 hrs.

Flow cytometry analysis. For cell cycle analysis, 1–2 x 10^6 cells were stained with propidium iodide. Briefly, the cells were washed twice in cold PBS and fixed in ice-cold 50% ethanol for 30 minutes. After two more washes in PBS, propidium iodide (Sigma Chemical Co., Poole, UK), and RNase A (Sigma) were added to a final concentration of 100 ng/mL each. After incubation for 1 hour at room temperature, the cells were kept at 4°C until analysis by flow cytometry (FACScan, Becton Dickinson, Temse, Belgium) using the CellQuest software.

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